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Breast cancer is the second leading cause of cancer-related death among women in the United States. Approximately 5-10% of breast cancer cases are associated with inheritable genetic factors. Two familial breast cancer susceptibility genes, *BRCA1* and *BRCA2*, have been isolated to date, with a third gene, *BRCA3*, still being actively sought. Mutations in *BRCA1* are associated with both ovarian and breast cancer, while mutations in *BRCA2* are associated with breast cancer in both women and men. It is not known why this difference occurs, but these findings may have important implications for the development of future surveillance, diagnosis, and treatment strategies of familial breast cancer. In keeping with the design of our original proposal, we have successfully generated a panel of monoclonal antibodies directed against various regions of the *BRCA2* protein as a result of three independent fusions. In addition, we have cloned the full-length *BRCA2* coding sequence into various mammalian expression vectors to study BRCA2 function in either transient or stable-inducible expression systems. We are now in the process of characterizing our antibodies and beginning experiments studying aspects of BRCA2's putative functions in both DNA-repair and transcriptional activation.

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ANNUAL SUMMARY
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INTRODUCTION

BRCA2 is the second of two breast cancer susceptibility genes discovered to date, and has been shown to be involved in both female and male hereditary breast cancer (HBC). Although the genetics of *BRCA2*-related HBC is well-characterized, very little is still known about the function of the protein product. This is due in part to the recent cloning of the gene, in which case a vast majority of early studies were focused upon identifying genetic mutations and alterations rather than studying protein function. However, the two main reasons it has been difficult to characterize the role of BRCA2 are its lack of significant sequence homology with other known proteins, and the lack of useful reagents. In order to begin addressing the issue of function, we had proposed to generate *BRCA2* fusion proteins and/or synthetic peptides to be used in immunizing Balb/c mice for the production of monoclonal antibodies directed against BRCA2 and to use these antibodies in various cellular and molecular studies in both transfected cell lines and tissues. In keeping with the design of the original proposal, we have since then generated a panel of monoclonal antibodies directed against various regions of the *BRCA2* protein as a result of three independent fusions. In addition, we have cloned the full-length *BRCA2* coding sequence into various mammalian expression vectors to study BRCA2 function in either transient or stable-inducible expression systems. We are now in the process of characterizing our antibodies and beginning experiments studying aspects of BRCA2's putative functions in both DNA-repair and transcriptional activation. Although some tissue studies will be pursued in this next phase of the project, the majority of the work will be performed on a BRCA2-inducible cell line, Capan-1 (pIND(SP1)-BRCA2FL), which can be induced to express barely-detectable to high levels of BRCA2. We anticipate that the use of this cell line, supported by follow-up data collected on actual frozen tissues, will yield novel discoveries that may help in our understanding of how BRCA2 functions during normal growth and development, and how loss of this function leads to an increased susceptibility to cancer.

YEAR 2 ANNUAL SUMMARY:

BODY

Expression and Purification of *BRCA2* peptides

In our Year 1 ANNUAL SUMMARY we described the generation and characterization of 2 monoclonal antibodies derived from our first fusion. These antibodies have been shown to be directed against the exon 10 region of BRCA2 as demonstrated by protein ELISA assay and western immunoblot. However, although these antibodies were specific for BRCA2, additional antibodies were required for the types of studies we were interested in pursuing. As a result, two additional fusions were prepared, one using a larger *BRCA2* fusion protein, containing exons 1-11, as the immunogen, and a second using a keyhole limpet hemacyanin (KLH)-conjugated synthetic peptide (Research Genetics, Inc.).

Exons 1-11 (BRCA2-FragI) were cloned from *BRCA2* complementary DNA (cDNA), which was reverse-transcribed from total RNA extracted from the HBL-100 breast cell line. This insert was cloned into a *pTrcHis2A* expression vector (Invitrogen) containing an in-frame carboxy-terminal *myc-His* epitope tag. The fusion protein was then expressed in BL-21 *Escherichia coli* and purified using nickel-nitriloacetate (Ni-NTA, Qiagen) agarose beads under denaturing conditions with 8 M urea. The column eluates were dialyzed against 0.1 M TrisHCl, 0.25 M NaCl, pH8.0, in step-wise decrements of urea concentration to facilitate re-folding to the closest approximation of the native conformation. The purified and renatured product was then resolved by SDS-PAGE and analyzed by immunoblotting using anti-His-tag or anti-myc-tag monoclonal antibodies (Invitrogen, La Jolla, CA) to confirm the purification of the appropriate fusion protein (Figure 1).

Synthetic peptides were purchased (Research Genetics, Inc.) as either unlabeled 20-amino acid molecules, or conjugated to a carrier molecule. For this protocol, the conjugated molecule was keyhole limpet hemacyanin (KLH).

Production of *BRCA2*-specific monoclonal antibodies

In order to minimize the likelihood of mixing hybridomas, Fusion #2 (BRCA2-FragI) and Fusion #3 (KLH-BRCA2) were done sequentially rather than simultaneously. Three female BALB/c mice were injected i.p. with 50 ug of appropriate protein mixed into an equal volume of complete Freund's adjuvant. Two booster injections were then given to each mouse two and four weeks later using incomplete Freund's adjuvant. Boosters were administered as a standard 50 ug i.p. dose, in addition to a 10 ug subcutaneous dose for prolonged exposure to immunogen. Four days prior to fusion date, all mice were given final boosts using PBS rather than adjuvant. On the day of the fusion, all mice that demonstrated immunoreactive sera by protein ELISA assay, or by western immunoblotting (Figure 2), were sacrificed by cervical dislocation under general anesthesia and their spleens removed by blunt dissection. The splenocytes were dissociated and fused with a Sp2/O-Ag14 myeloma cell line in the presence of PEG. Hybridomas were selected for using HAT media supplementation (Hypoxanthine, Aminopterin, Thymidine). HAT selection was maintained for 14 day post-fusion, and subsequently removed once all the unfused spleen and myeloma cells had been selected against. Hundreds of distinct hybridoma colonies were detected by Days 12-20. Each hybridoma colony was screened for antibody production every 3 days by ELISA against either purified BRCA2-FragI recombinant protein, or unconjugated BRCA2 synthetic peptide. For Fusion #2, duplicate ELISA assays were performed using a non-specific, bacterially-expressed and purified protein as a subtractive negative control. An arbitrary cut-off was used to screen for the hybridoma colonies with the most specific BRCA2 antibodies, as monitored using the visual chromogen, OPD, and read by an ELISA plate reader. These high expressors were cultured and frozen stocks were made by pelleting down the cells and resuspending in a 10% mixture of DMSO in culture media. A few of the high expressors for both Fusion #2 and Fusion #3 were maintained and subcloned twice by limiting dilutions in order to isolate monoclonal hybridomas. Each round of subcloning was accomplished by plating 96-well culture plates with a low density of cells (1 hybridoma/well) and expanding wells with continued high expression of BRCA2-specific antibodies. Table 1 lists characteristics of each monoclonal antibody generated from Fusion #2 and Fusion #3, as well as Fusion #1.

Immunohistochemistry on Frozen Tissues for BRCA2 Localization

Monoclonal antibodies from Fusion #2 have been used to determine the cellular distribution of BRCA2 in frozen sections of various breast cell lines. MCF-7, HBL-100, and Capan-1 cells lines were grown, trypsinized, and pelleted in freezing compound (OCT). Frozen sections of 4-5 microns thick were cut on a cryostat and mounted onto glass slides for immunohistochemical analysis. Sections were exposed to 0.5% H_2O_2 to wash out any endogenous peroxidase activity and then blocked in a 10% solution of rabbit serum (PBS). After blocking, each slide was incubated with hybridoma supernatents for 1-2 hours before being rinsed in PBS and incubated with secondary (rabbit anti-mouse) and tertiary (mouse PAP) antibodies (1:50 dilution each). Immune complexes were detected using a chemical detection system (3,3'diaminobenzidine). Slides were then counterstained with ethyl green, a nuclear stain, dehydrated, and permanently mounted with Permount (Fisher). Table 2 shows a summary of the type and intensity of staining for Fusion #2 monoclonal antibodies. Fusion #3 monoclonal antibodies are currently in the process of being characterized on frozen and paraffin sections.

Cloning the Full-Length *BRCA2* from HBL-100 complementary DNA\

Full-length *BRCA2* was constructed from the ligation of two overlapping PCR fragments amplified from reverse-transcribed mRNA derived from the HBL-100 breast cell line. In order to decrease the potential for the introduction of random, PCR-generated errors, a deep-vent polymerase was used (Gibco-BRL). Figure 3 demostrates the strategy for cloning and also lists the pairs of overlapping primers used for cloning. In brief, cDNA from HBL-100 was used as the template in 50 uL reaction volumes containing 1.7mM magnesium sulfate, .2 mM dNTPs, 1X reaction buffer, and appropriate primers. Reactions were gel-purified (Qiagen) and cloned into shuttle vectors (TA Cloning, Invitrogen). FRAGI-II (Exons 1-11) was amplified using primer pairs PR643 and PR640, whereas FRAGIII (Exons 11-27) was amplified using primer pairs PR639 and PR638. FRAGI-II has an engineered 5' KpnI site and an engineered 3' SalI site. FRAGIII contains an overlapping 5'SalI site, as well as an engineered 3' XhoI site. Below lists the sequences of the two primer pairs used in this portion of the study.

PCR Primers
(FRAGI-II), 212 bp - 6922 bp
PR643 (5' GAATATCGGTACCGTAGGTAAAAATGCCTATTGG 3')
PR640 (5' TAGCAATGTCGACTGCTTCTGTTTCAAAGTAGTT 3')
(FRAGIII), 6903 bp - 10502 bp
PR639 (5' AGAAGCAGTCGACATTGCTAAAGCTTTTATGGAA 3')
PR638 (5' GTCGCCTCTCGAGTTGCAAATGCTTAGATATATT 3')

Generation of *BRCA2* Mammalian Expression Vectors

Capan-1 cells have been shown to contain a truncation mutation (6173delT) in one copy of the *BRCA2* gene, and loss of the other wild-type copy. Immunohistochemical analysis of this cell line using our Fusion #2 clones show low to barely-detectable levels of staining, whereas a positive control antibody directed against BRCA1 (AB-1, Oncogene Research) show dark, nuclear staining. Because these cells express only low levels of truncated protein, this cell line is the optimal system to study BRCA2 interactions and function. In order to pursue these studies, two different expression vectors were generated using a full-length copy of the *BRCA2* coding sequence. For transient-expression studies, we have cloned the *BRCA2* coding sequence into a CMV-driven pcDNA3.1/*myc*HisC(-) mammalian expression vector (Invitrogen) using novel NotI/KpnI restriction sites. The *BRCA2* insert contains its own Kozak sequence and end-termination site. In addition to our own *BRCA2* clone, full-length *BRCA2* was generously provided by Dr. Wen-Hwa Lee at the University of Texas Health Science Center.

For the generation of a stable, inducible *BRCA2* cell line, the pIND(SP1)-Ecdysone-Inducible Mammalian Expression system (Invitrogen) was selected. This system allows for tightly-controlled regulation of expression using an insect hormone (ecdysone) response element that can activate transcription when bound by its ligand-associated receptor (VgEcR/RXR). Because this is an inducible system that requires co-expression of a regulatory protein (VgEcR/RXR), two vectors were required to be stably-transfected in cells. The receptor-containing plasmid has a Zeocin-selection marker and the expression vector has a Neomycin selection marker. Capan-1 cells were titrated for Zeocin and G418 cytotoxicity in preparation for stable selection. We are currently in the process of isolating stable co-transfectants containing both receptor and expression plasmids in the presence of Zeocin and G418. We anticipate isolating inducible clones in the near future.

APPENDICES

A. Figures and Tables

Figure 1. Western Immunoblot of Recombinant BRCA2-FragI Protein. Recombinant BRCA2-FragI proteins were affinity-purified, subjected to SDS-PAGE and transfered onto nitrocellulose membranes by either wet or semi-dry transfer methods. Membranes were blocked overnight in 5% milk solution and then incubated for 1-2 hours with either anti-His antibody (Qiagen) or anti-myc antibody (Invitrogen). Membranes were then washed with PBST and incubated with a 1:2000 dilution of goat anti-mouse secondary antibodies (Biorad) for 1 hour. Antibody complexes were detected with the ECL chemiluminescent system (Amersham).

Figure 2. Western Analysis of Mouse Sera Derived from BRCA2-FragI Immunized Balb/c Mice. As described in Figure 1, purified proteins were resolved by SDS-PAGE and transfered onto nitrocellulose membranes. Membranes were blocked overnight in 5% milk solution and then incubated for 1-2 hours with a 1:200 dilution of immune sera in 1% milk. Membranes were then washed with PBST and incubated with a 1:2000 dilution of goat anti-mouse secondary antibodies (Biorad) for 1 hour. Antibody complexes will be detected with the ECL chemiluminescent system (Amersham).

Figure 3. Cloning of BRCA2 Using Long PCR Amplification and Overlap Extension. As depicted in the diagram, *BRCA2* was amplified as two overlapping fragments using long PCR techniques. The two overlapping fragments were subsequently cloned into a TA Cloning shuttle vector for further manipulations.

Table 1. Summary of Monoclonal Antibodies. Table 1 lists all monoclonal antibodies derived from three separate fusion experiements. Also listed are all relevant assays and procedures performed using these antibodies during initial characterization studies.

Table 2. Immunohistochemical Results of Fusion #3 Monoclonal Antibodies. The staining intensities are defined as a range from (+) = weak to (+++) = very strong, or (-) = negative.

FIGURE 1.

Pilot Expression/Purification of BRCA2-FragI

Purified BRCA2-FragI Recombinant Protein

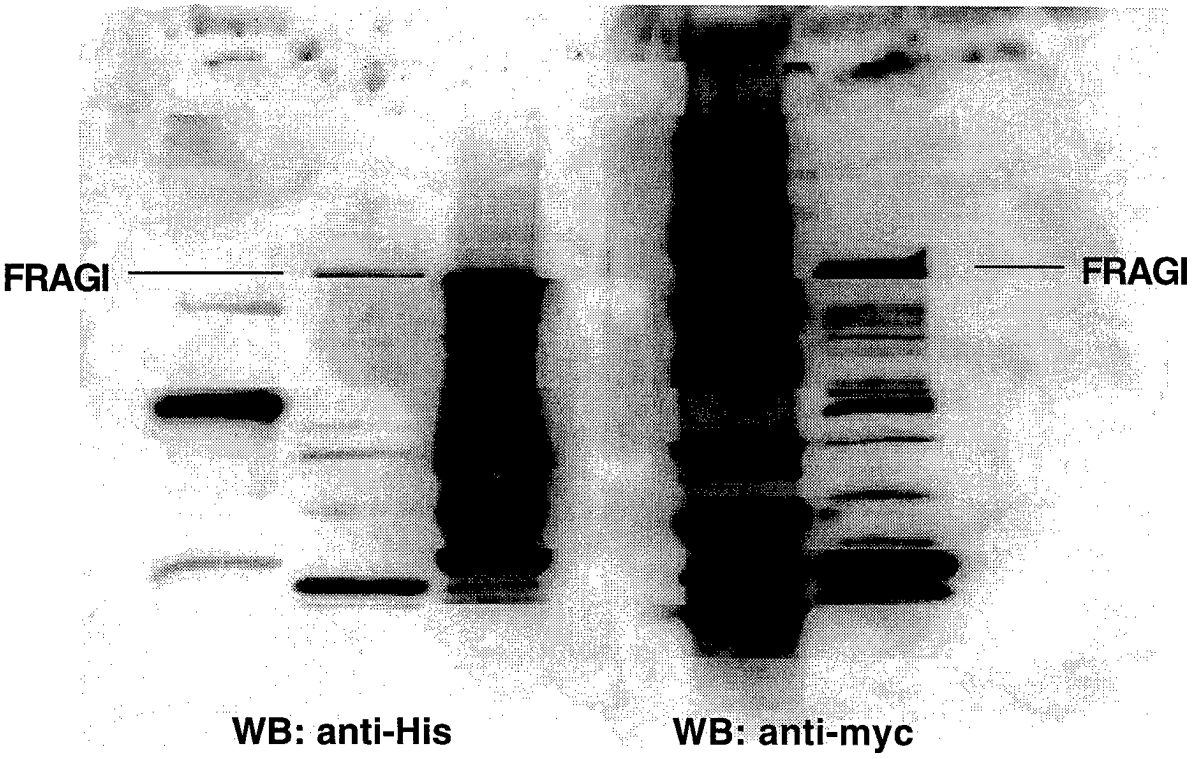
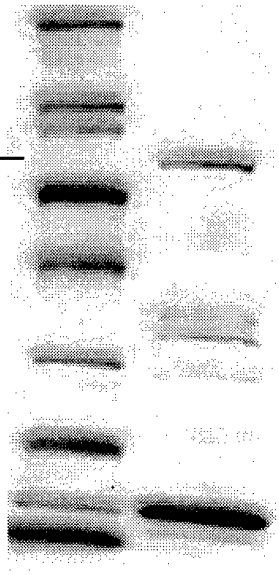


FIGURE 2.

Polyclonal Sera: BRCA2-FragI

Mouse #3 (of 3 immunize mice)

A)



WB: anti-myc mAb

B)

WB: Sera #3 (1:200 in PBST)

FIGURE 3.

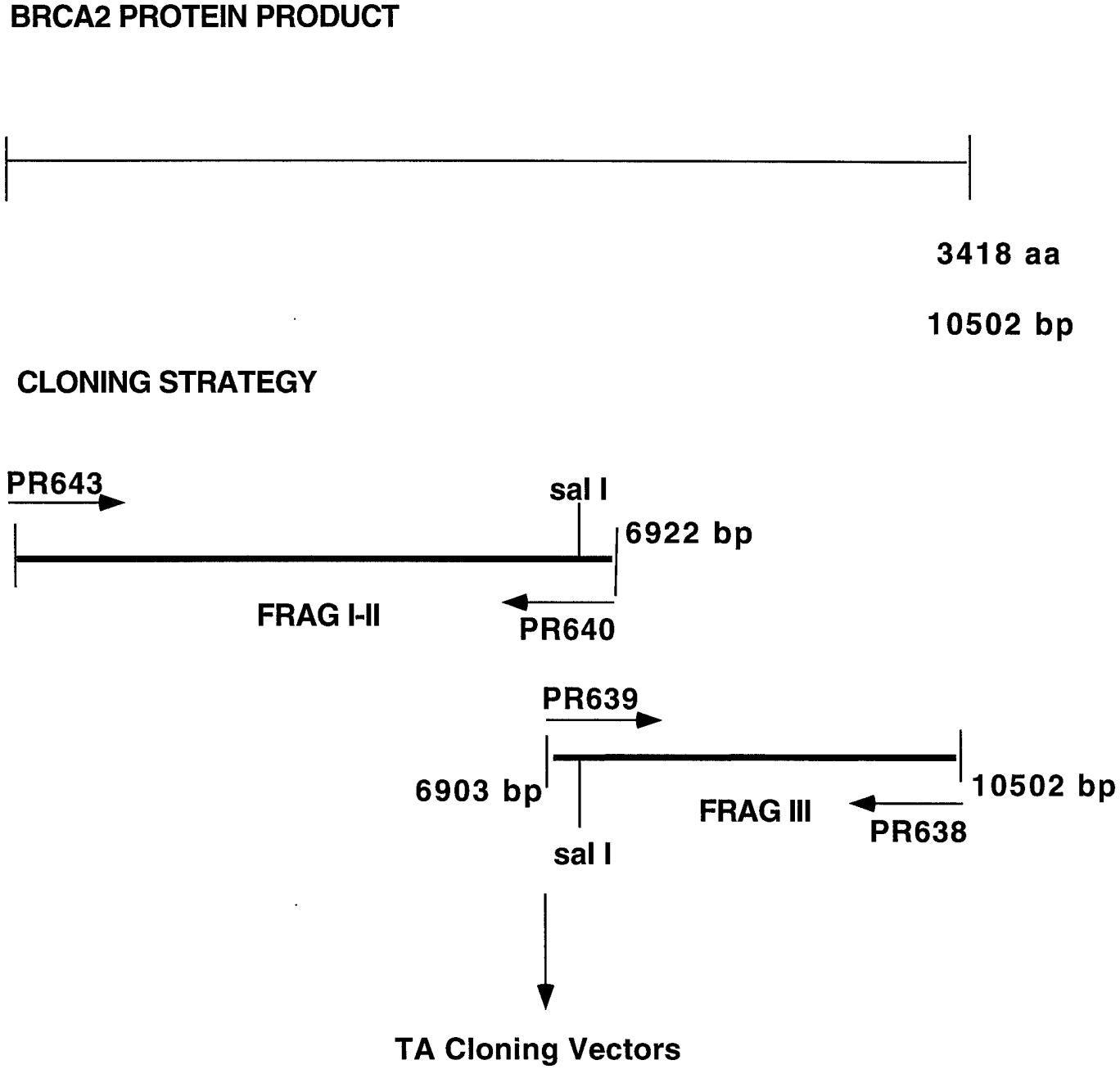

BRCA2 PROTEIN PRODUCT
3418 aa
10502 bp
CLONING STRATEGY
PR643
sal I
6922 bp
FRAG I-II
PR640
PR639
6903 bp
FRAG III
10502 bp
PR638
sal I
TA Cloning Vectors

TABLE 1.

Monoclonal Antibody	Protein ELISA	Western Immunoblotting	IHC
Fusion #1			
9H1	Y	Yes	N/A
8D1	Y	Yes	N/A
Fusion #2			
7B12.H9.H6	Y	Yes	(+++)
12F1.A8.C6	Y	Yes	(+++)
2F4b.D5.E9	Y	Yes	(++/+++)
10D8.C1.G10	Y	Yes	(+++)
Fusion #3			
4D12.E9.G12	Y	N/A	N/A
3E1.F11.H7	Y	N/A	N/A
2D5.H5.G12	Y	N/A	N/A
2C8.F8.G1	Y	N/A	N/A
1G8.G7.H11	Y	N/A	N/A
2E5.H8.G12	Y	N/A	N/A

TABLE 2. Fusion #2 Immunohistochemistry

Monoclonal Antibodies	IHC	Cellular Location
7B12.H9.H6	(+++)	Nuclear
12F1.A8.C6	(+++)	Nuclear
2F4b.D5.E9	(++/+++)	Nuclear
10D8.C1.G10	(+++)	Nuclear

B. Additional Information

1) Key Research Accomplishments:

- Multiple monoclonal antibodies from three independent fusions have been isolated, subcloned and preliminarily characterized for BRCA2-specificity.

- Full-length *BRCA2* has been cloned by long PCR techniques involving amplification and cloning of overlapping regions of the coding sequence.

- A stable, inducible cell line expressing full-length BRCA2 is currently being made using an Ecdysone-Inducible Mammalian Expression System (Invitrogen). Once fully-isolated and subcloned, it will be called Capan-1 (pIND(SP1)-BRCA2FL).